Sarcomeric function and protein changes in human cardiomyopathy

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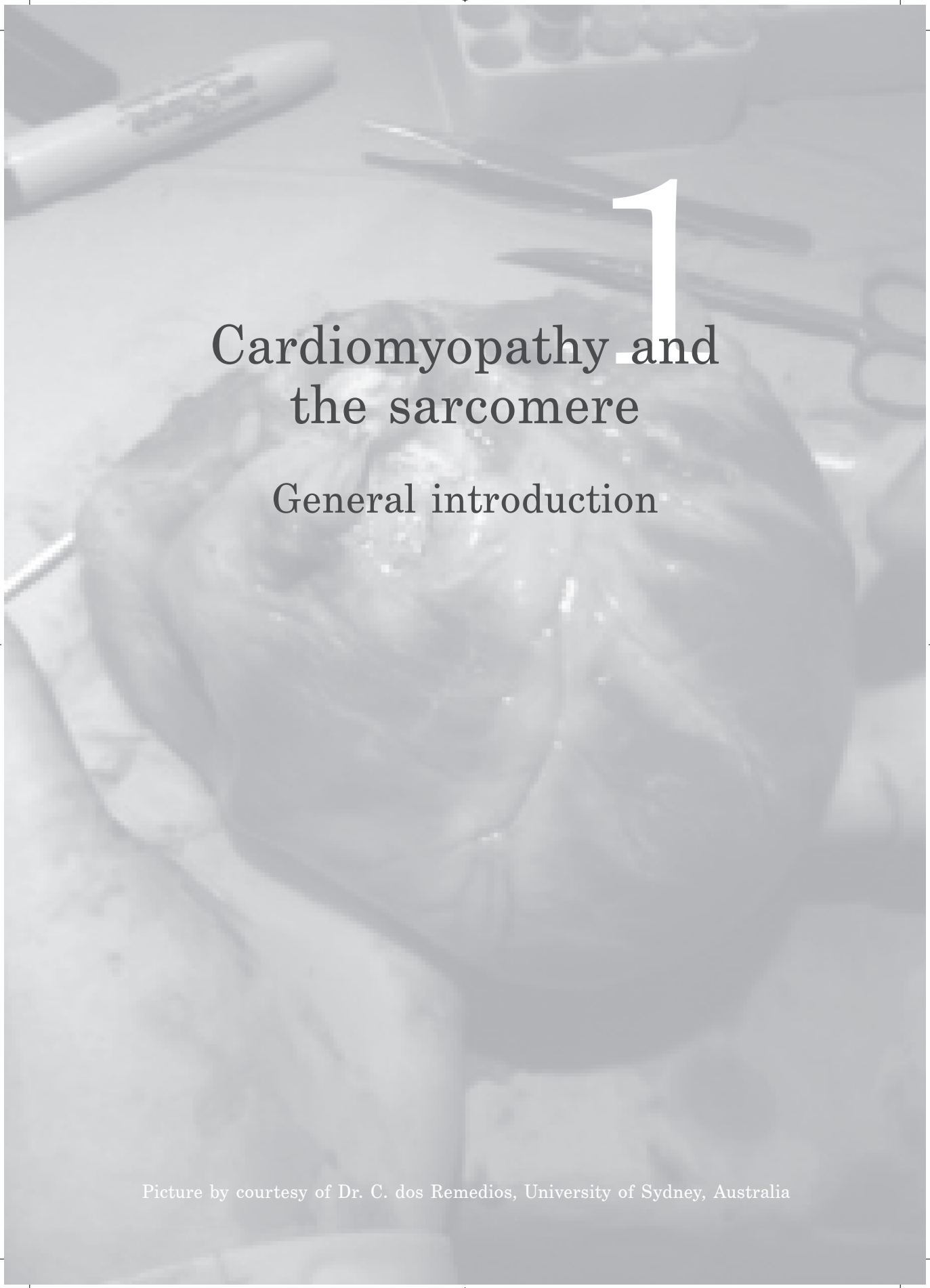

1 Cardiomyopathy and the sarcomere

General introduction

Picture by courtesy of Dr. C. dos Remedios, University of Sydney, Australia

CLASSIFICATION OF CARDIOMYOPATHIES

Heart failure is the general term for the inability of the heart to supply sufficient blood to the body in patients suffering from cardiac disease. The symptoms range from no limitation in physical activity to the inability to perform any physical activity at all and patients are divided in four subgroups by the New York Heart Association (NYHA class I: cardiac disease without limitation of physical activity to NYHA class IV: heart failure present even at rest).[1]

Cardiac diseases can have an external cause, such as coronary artery disease, valve disease or hypertension, or may involve cardiomyopathies, in which the heart muscle itself is abnormal (i.e. an intrinsic cause of the disease is present in the heart muscle). The distinction between different classes of cardiac diseases is an important one to make, as cardiac diseases with similar phenotypes can have a diverse origin and may need different types of management. However, classification of cardiomyopathies is difficult, as the origin or pathophysiology is not always understood. Furthermore, at present there is no consensus on how to classify cardiomyopathies (e.g. based on origin, physiology or treatment) among clinicians.[2;3]

In order to promote an uniform nomenclature and well-defined clinical patient groups, recent knowledge on underlying causes and pathophysiology of cardiomyopathies has been implemented in a cardiomyopathy classification system both on behalf of the American Heart Association (AHA) and of the European Society of Cardiology (ESC).[2;3] The AHA predominantly focuses on the principal organ involved and divides cardiomyopathies in *primary* when the heart is the sole or predominantly affected organ and in *secondary* when the cardiomyopathy is part of a systemic disorder. Primary cardiomyopathies are further divided in genetic, acquired or mixed, depending on the main cause of disease.[2] The ESC guidelines are more clinically orientated, which is appealing as this circumvents the complex pathophysiology of cardiomyopathies, which is not always comprehended upon presentation of the patient. According to the ESC guidelines cardiomyopathies are classified as hypertrophic (HCM), dilated (DCM), restrictive (RCM), arrhythmogenic right ventricular (ARVC) or unclassified cardiomyopathy based on morphological and functional phenotypes (Figure 1.1). Each phenotype has a familial (genetic) or non-familial (non-genetic)

sub-classification. Cardiomyopathies are classified as familial when the same disorder or phenotype occurs in more than one family member and is (or can be) caused by the same genetic mutation. In familial cardiomyopathies a distinction is made between those patients in whom the mutation is detected and patients with (yet) unknown (unidentified) genetic defects. Also non-genetic cardiomyopathies can be distinguished in a similar way: those with an unknown etiology (idiopathic) and acquired cardiomyopathy (such as inflammatory or metabolic cardiomyopathies, neuromuscular disorders or toxic reactions).[3;4]

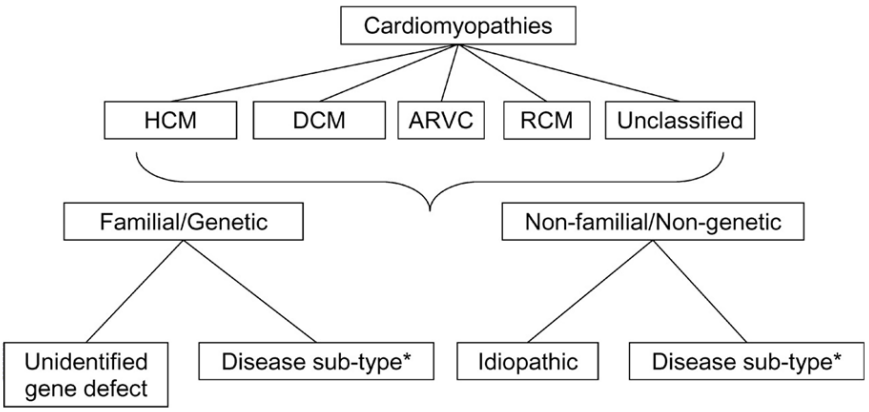

Figure 1.1. Summary of the classification system adapted from the European Society of Cardiology.[3] Cardiomyopathies are grouped based on functional and morphological clinical characteristics. Each group can be subdivided in familial or non-familial and within these groups the defect/etiology may or may not be known. HCM indicates hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; ARVC, arrhythmogenic right ventricular cardiomyopathy; RCM, restrictive cardiomyopathy. *Disease sub-type refers to different cardiomyopathies with a known etiology.

HYPERTROPHIC AND DILATED CARDIOMYOPATHY

Genetic factors involved in heart failure are becoming increasingly known, in particular in HCM and DCM. At present over 900 mutations in 13 genes have been identified to cause HCM and 15 causal genes are described for DCM.[5;6] However, it is still largely unknown by which mechanisms genetic mutations eventually lead to cardiomyopathy.

DCM is characterized by an increased left ventricular (LV) volume and a reduced ejection fraction (systolic failure). The prevalence of DCM is about 1 in 2500 and 25-30% of the DCM cases is classified as familial, indicating

the presence of a mutation in these patients.[5;7;8] Although the cause is often unknown, a number of mutations, some of which are located in the sarcomeric proteins, have been found to cause DCM.[5;8]

HCM has a prevalence of 1:500 and is characterized by asymmetrical thickening of particularly the interventricular septum (IVS) and LV wall without an underlying cardiac or systemic defect that could account for the degree of hypertrophy (Figure 1.2).[3;9] HCM is a heterogeneous disease with a wide range of genetic defects and clinical presentations. The causative mutation most often lies in genes encoding several sarcomeric proteins and is found in about 60% of the HCM patients (Table 1.1).[6;10;11] Familial HCM (with our without identified gene defect) is the most frequent inheritable cardiac disease, and approximately two-thirds of HCM patients have a family history.[12;13] Clinical presentation is very diverse, ranging from virtually no complaints despite cardiac hypertrophy to sudden cardiac death in patients with limited thickening of the heart. Noteworthy, even in patients carrying the same disease-causing mutation clinical presentation is variable and it is impossible to link clinical outcome to underlying mutations.[5;14-16] Recently, a long-term follow up identified three clinical profiles among patients with manifest HCM based on the predominant pathophysiological disease component: (i) end-stage systolic dysfunction, (ii) LV outflow tract obstruction at rest (Figure 1.2) and (iii) non-obstructive with preserved systolic function (i.e. diastolic dysfunction).[17]

Even before clinical features of HCM are apparent in individuals carrying a HCM mutation, signs of cardiac dysfunction may be observed. Subtle diminishment of strain velocities during early diastole was observed with echocardiographic strain analysis and cardiovascular magnetic resonance imaging in mutation carriers compared to healthy individuals, indicating diastolic dysfunction in the preclinical stage of HCM.[18-20] Furthermore, profibrotic markers were found in the serum of preclinical HCM mutation carriers, preceding visual evidence of fibrosis characteristic in overt HCM.[21] Preclinical cardiac dysfunction indicates that the hypertrophic phenotype might merely be a secondary, though detrimental, adaptation to cardiomyocyte dysfunction.

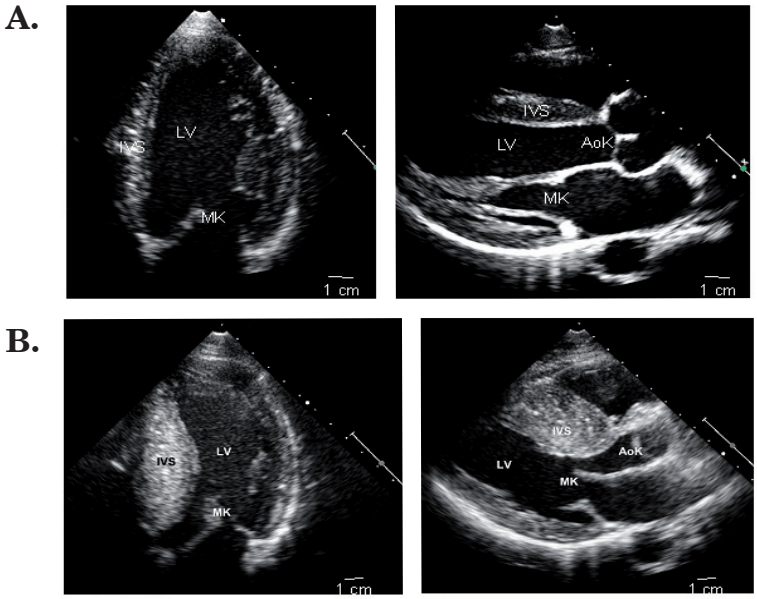

Figure 1.2. Echocardiographic images from a healthy person and a hypertrophic cardiomyopathy patient. On the left a four-chamber view focussed on the left ventricle and septum. The septum of a HCM patient **(B)** is considerably thicker than the septum of a healthy control **(A)**. On the right a parasternal long axis view, visualizing outflow tract obstruction by the hypertrophied septum in a HCM patient **(B)**. In healthy persons **(A)** the septum does not occlude the outflow tract from the left ventricle to the aorta. IVS indicates interventricular septum; LV, left ventricle; MK, mitral valve; AoK, aortic valve. Pictures by courtesy of Dr. F.J. ten Cate, Erasmus Medical Center Rotterdam, the Netherlands.

Table 1.1. Genetic causes of hypertrophic cardiomyopathy.[10;11]

Gene	Symbol	Frequency
β-Myosin heavy chain	*MYH7*	25-40%
Myosin binding protein C	*MYBPC3*	25-40%
Troponin T	*TNNT2*	~5%
Troponin I	*TNNT3*	~5%
α-Tropomyosin	*TPM1*	1-2%
Myosin light chain 1	*MYL3*	~1%
Myosin light chain 2	*MYL2*	Rare
α-Actin	*ACTC1*	~1%
Titin	*TTN*	Rare
α-Myosin heavy chain	*MYH6*	<1%
Muscle LIM protein	*CSRP3*	Rare
Telethonin	*TCAP*	<1%
Troponin C	*TNNC1*	Rare

CARDIAC MYOCYTE CONTRACTILITY: THE SARCOMERE

The contractile properties of the sarcomere, the smallest functional contractile unit of cardiac muscle, are important determinants of cardiac pump function. Main components of the sarcomere are the thick and the thin filament, respectively composed of myosin and actin. The cardiac sarcomere contains many more proteins, such as troponins I, T and C (cTnI, cTnT and cTnC), tropomyosin (TM), myosin binding protein C (cMyBP-C) and titin, that control the process of contraction and relaxation (Figure 1.3).

A rise in intracellular free calcium upon activation of the cardiac cell initiates contraction by binding of calcium to cTnC. As a result, the tropomyosin-troponin complex changes its conformation, thereby unblocking myosin binding sites on actin and allowing myosin heads extending from the thick filament backbone to bind to actin, i.e. cross-bridge formation. Then, the myosin head rotates upon release of adenosine diphosphate (ADP) and inorganic phosphate (Pi), when the energy stored in the myosin head is released. Since myosin is attached to actin, this rotation causes sliding of the actin filament past the myosin filament, the so-called power stroke. The subsequent binding of adenosine triphosphate (ATP) to myosin induces detachment of the myosin head from actin, completing the cross-bridge cycle.[23;24] Cross-bridge cycling, e.g. the number, cycling rate and force produced per cross-bridge, is modulated by interplay of all sarcomeric proteins, including cMyBP-C (reviewed by de Tombe[25]).

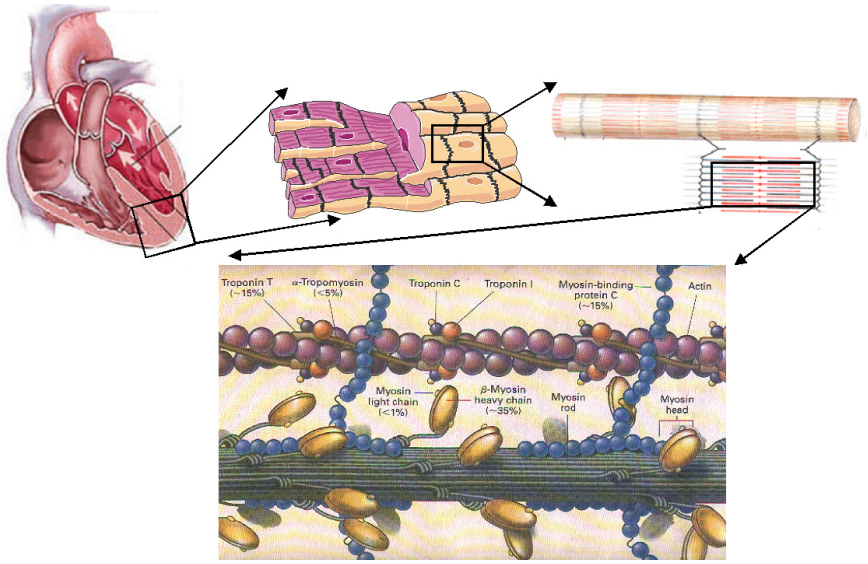

Figure 1.3. The heart contains striated muscle cells, which consist of bundles of myofibrils. Myofibrils are composed of many sarcomeres organised in series. A sarcomere consists of the thin and the thick filament.[22] The thin filament is composed of actin, associated with tropomyosin and the troponin complex (troponin I, T and C). The thick filament mainly consists of myosin (built up of myosin heavy and light chains) and is accompanied by myosin binding protein C. Titin forms a third filament, which spans the half sarcomere fromits border, the Z-line, to the middle, its M-line (not depicted in this figure). The percentages in the lower panel give the estimated frequency with which a mutation in the corresponding gene cause HCM.[22]

CARDIAC MYOSIN BINDING PROTEIN C

One of the most commonly affected genes in hypertrophic cardiomyopathy encodes the thick filament protein cMyBP-C.[26;27] The sarcomeric protein cMyBP-C is located in the C-zone of the A-band, in 7 to 9 transverse stripes located 43 nm apart (Figure 1.4A).[28;29] cMyBP-C consists of 8 immunoglobin-like and 3 fibronectin domains numbered C0 to C10, with binding sites for myosin, actin and titin (Figure 1.4B). The N-terminal C0 is specific for the cardiac isoform of MyBP-C, as is the MyBP-C like motif between C1 and C2, which contains 3 phosphorylation sites. Third, the cardiac isoform has 28 additional residues in the C5 domain compared to the skeletal isoform of MyBP-C.

The function of cMyBP-C is not fully understood, though it has both structural and regulatory roles. cMyBP-C contributes to sarcomeric stability in vitro,[30;31] though it is not essential for correct assembly of sarcomeres as

evident from the viability of cMyBP-C null-mice.[32;33] Functionally, cMyBP-C appears to fine-tune the timing of contraction. cMyBP-C limits cross-bridge cycling rate,[34;35] by tethering the myosin heads close to the backbone and thereby impairing cross-bridge formation[36;37] or it may act as an internal load, slowing down the cross-bridges.[38;39] Ablation of cMyBP-C in a cMyBP-C null-mouse led to a marked abbreviation of systolic ejection[40] and faster cross-bridge kinetics.[34;41]

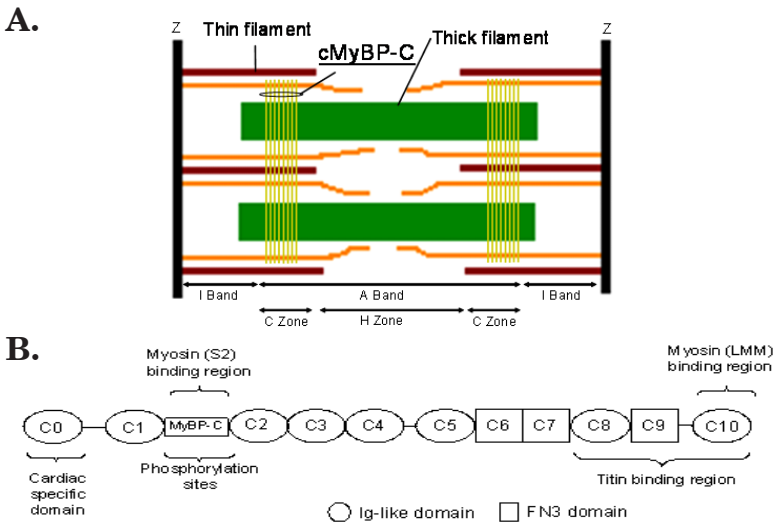

Figure 1.4. Schematic representation of a muscle sarcomere and cardiac myosin binding protein C. A. Outline of a sarcomere depicting cMyBP-C as transverse stripes in the C-zone. **B.** cMyBP-C consists of domains C0 to C10, with the cardiac specific C0 domain at the N-terminus and cardiac specific (protein kinase A) phosphorylation sites in the MyBP-C like motif.

ROLE OF SARCOMERIC PROTEIN PHOSPHORYLATION IN CARDIAC FUNCTION

Post-translational modifications, in particular phosphorylation, regulate the function of several cardiac sarcomeric proteins (for a recent review, see Jin *et al.*[42]). The foremost kinases active in the heart are protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}-dependent calmodulin kinase 2 (CamK2). Several studies have reported a downregulation of PKA and an up-regulation of PKC and CamK2 in heart failure.[43-46] Dephosphorylation of sarcomeric proteins is regulated by phosphatases like protein phosphatase 1 and protein phosphatase 2A, which activities are increased in cardiac disease.[47] Changes in

kinase and phosphatase expression/activity may alter phosphorylation of the sarcomeric proteins in cardiac disease and impair sarcomeric function.[48]

PKA is activated upon β-adrenergic receptor stimulation (Figure 1.5) and is a key player in cardiac adaptation to increase cardiac demand as occurs during stress or exercise. The main sarcomeric protein targets of PKA-dependent phosphorylation are cTnI, cMyBP-C and titin. PKA-mediated phosphorylation of titin reduces passive tension in cardiomyocytes.[49;50] Phosphorylation of cTnI by PKA is associated with reduced Ca^{2+}sensitivity of force and increased cross-bridge kinetics, enabling faster relaxation to maintain adequate diastolic function at elevated cardiac output.[44;48] Evidence suggests a role for PKA-dependent activation of cMyBP-C in the kinetics of force development. Phosphorylation of cMyBP-C by PKA relieves its interactions with other sarcomeric proteins,[37;51] promoting cross-bridge cycling. Indeed, treatment of skinned ventricular myocardium with PKA accelerated stretch activation in mice, an effect that could specifically be ascribed to cMyBP-C phosphorylation.[52] A transgenic mouse model in which the phosphorylation sites of cMyBP-C were replaced by non-phosphorylatable alanines demonstrated decreased contraction and relaxation of the heart, implying the necessity of cMyBP-C phosphorylation for proper cardiac function.[53]

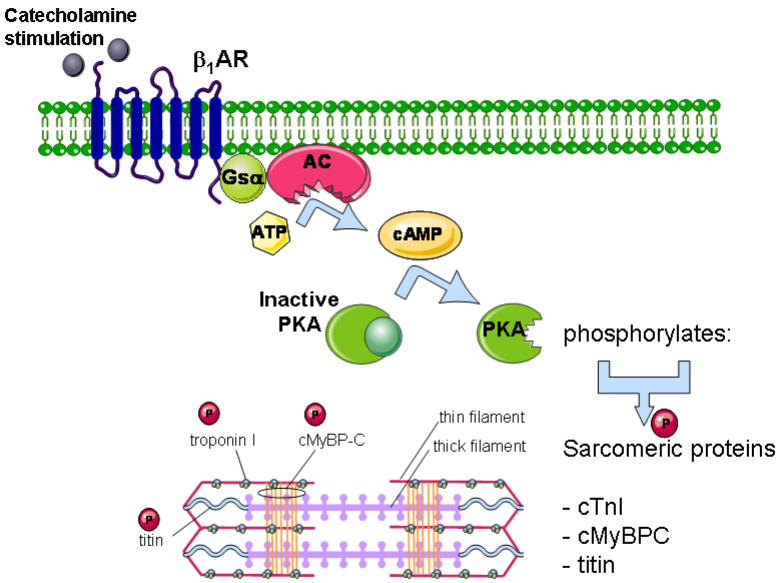

Figure 1.5. Overview of the β-adrenergic receptor pathway. Activation of the β1-adrenergic receptor (β1AR) by catecholamines, such as (nor)adrenaline, activates adenyl cyclase (AC) via the stimulatory G-protein (Gsα). AC synthesizes the second messenger cyclic adenosine monophosphate (cAMP) from ATP. cAMP can activate protein kinase A (PKA) which phosphorylates, among others, the sarcomeric proteins cardiac troponin I (cTnI), myosin binding protein C (cMyBP-C) and titin.

AIM AND OUTLINE OF THE THESIS

To gain better insight in the pathophysiology of cardiomyopathies and of myocyte function in general, further investigation of changes in morphology, biochemical profile and genetics of patients is required. The spectrum of underlying mutations is wide in HCM and its clinical presentation is very heterogeneous. Furthermore, the spectrum of mutations causing DCM overlaps with those causing HCM, yet the clinical phenotype of DCM and HCM is substantially different. The pathophysiological process leading from a pathogenic mutation to cardiomyopathy or what distinguishes the progression to either HCM or DCM in particular, is largely unknown. Consequently, at present no specific therapy is available to prevent or reverse cardiac remodelling and dysfunction in primary cardiomyopathies.

In this thesis studies are described aimed to make a distinction between the unique consequences of mutations (focused on the *MYBPC3* gene, encoding cMyBP-C) and pathological changes and compensatory mechanisms

of cardiomyopathies. Figure 1.6 depicts the main topics of the studies described in this thesis.

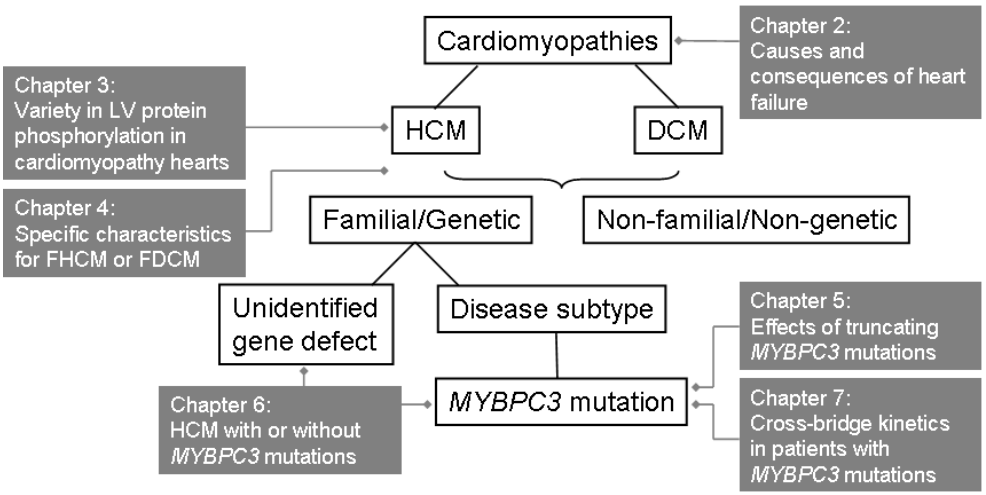

Figure 1.6. Adaptation of the classification system proposed by the working group on myocardial and pericardial diseases of the European Society of Cardiology,[3] with the studies described in this thesis depicted in the grey boxes.

The next chapter, **chapter 2** gives an overview of the alterations observed in the failing heart and the factors that may contribute to impaired cardiac function, such as morphological changes, biochemical alterations and genetic defects. In addition, the roles of the sarcomeric proteins cTnI and cMyBP-C and their phosphorylation in health and disease are described.

Research on human cardiac tissue is greatly hampered by the availability of tissue. During cardiac surgery only small samples can be taken from just one region of the heart. However, regional variation in protein composition has been reported and may be of particular interest in a heterogeneous disease like HCM. In **chapter 3** we report on how representative small LV biopsies are for the region they were taken from. Thereto, we determined if local differences in sarcomeric protein phosphorylation are more evident in familial HCM or DCM than in non-failing donor samples.

Both DCM and HCM may be caused by mutations in several genes encoding sarcomeric proteins. At present it is not possible to predict clinical outcome on the basis of the location of a mutation. Moreover, the pathophysiological mechanisms leading to either DCM or HCM are not well understood. In

chapter 4 we compare sarcomeric function and protein composition between end-stage failing DCM and HCM cardiac samples to assess which alterations are specific for the particular cardiac phenotype and which changes are common to the failing heart in general.

The focus of **chapter 5** is on HCM caused by mutations in the *MYBPC3* gene encoding cMyBP-C. The frequent occurrence of founder mutations in the *MYBPC3* gene in the Netherlands enabled us to study the effect of these specific truncating *MYBPC3* mutations on mRNA and protein expression, sarcomere phosphorylation and function in a rather homogenous patient group.

In continuation of the results described in chapter 5, we examined which characteristics in sarcomeric protein composition and function can specifically be ascribed to *MYBPC3* mutations and which are common to familial HCM without identified sarcomeric mutations in **chapter 6**. Thereto, we examined cardiac samples from patients harbouring a *MYBPC3* mutation compared to HCM patients without a *MYBPC3* mutation and non-failing donors. Functional response of cardiomyocytes to an increase in length (the Frank-Starling mechanism) and to PKA (i.e. β-adrenergic receptor stimulation), two sarcomere response mechanisms important during increased cardiac demand, were assessed.

Chapter 7 describes the role of cMyBP-C in cross-bridge kinetics. We compared the rate of tension redevelopment after disruption of all cross-bridges upon a rapid decrease in length in cardiomyocytes from HCM patients with a *MYBPC3* mutation to non-failing donor cardiomyocytes. Furthermore, we measured cross-bridge kinetics from the response in force to a small stretch of the cardiomyocytes.

The thesis is concluded with a summary of all major findings of these studies and a general discussion in **chapter 8**.